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Inturned Localizes to the Proximal Side of Wing Cells under the Instruction of Upstream Planar Polarity Proteins

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Summary

Planar polarity development in the *Drosophila* wing is under the control of the *frizzled* (*fz*) pathway [1–3]. Recent work has established that the planar polarity (PP) proteins become localized to either the distal, proximal, or both sides of wing cells [4–9]. Fz and Dsh distal accumulation is thought to locally activate the cytoskeleton to form a hair [4, 10, 11]. Planar polarity effector (PPE) genes such as *inturned* (*in*) are not required for the asymmetric accumulation of PP proteins, but they are required for this to influence hair polarity [1, 2, 8, 9, 12–14]. *in* mutations result in abnormal hair polarity and are epistatic to mutations in the PP genes [12–15]. We report that In localizes to the proximal side of wing cells in a PP-dependent and PP-instructive manner. We further show that the function of two other PPE genes (*fuzzy* and *fritz*) is essential for In protein localization, a finding consistent with previous genetic data that suggested these three genes function in a common process. These data indicate that accumulation of proteins at the proximal side of wing cells is a key event for the distal activation of the cytoskeleton to form a hair.

Results

In Localizes to the Proximal Side of Pupal Wing Cells

We examined the subcellular distribution of the In protein by immunofluoresence. In did not show a distinctive subcellular distribution in third instar imaginal wing discs or in prepupal wings. In 27 hr pupal wings, we could reliably detect the endogenous In protein accumulating in a zigzag pattern that resembled that seen for PP proteins (Figure 1A). In favorable preparations, we could detect this pattern in both younger (24 hr) and older (32 hr) pupal wings. We assessed the relative level of In along the proximal/distal versus the anterior/posterior sides of wing cells in two ways. In one, we simply measured and compared the staining intensity along the two sides. The staining ranged from 1.2- to 1.7-fold higher along the proximal/distal edges of cells. This approach is likely to underestimate the asymmetric accumulation because of not correcting for the presence of nonspecific background staining. To correct for nonspecific background, we measured the intensity of staining away from the cell periphery and subtracted this from both proximal/distal and anterior/posterior values. This correction lead to estimates of 3.0- to 3.5-fold higher staining at the proximal/distal edge than the anterior/posterior edge. These estimates flank the range of estimates reported for PP proteins such as Fz (for example, see [8] and [11]).

The accumulation of planar polarity proteins such as Fz is uneven along the proximal/distal side of cells [4–9, 16]. This was also true for In. In experiments where we localized both Fz and In, we found that the uneven distribution of these proteins was usually coordinated so that membrane regions with a high concentration of one also had a high concentration of the other (Figures 1A–1F). This is also the case when two PP are localized and suggests that the In protein was being localized in response to the accumulation of one or more of the planar polarity proteins. In Z sections, we found that In was localized apically at the same apical-basal level as Fz (Figures 1G, 1H, and 1I) [8, 17].

To determine if the In protein was localized at the proximal or distal (or both) sides of cells, we stained wings that contained *in* clones marked by the loss of GFP (Figures 2A and 2B). We found that In was localized at the proximal side of wild-type cells located at the distal border of *in* clones. In contrast, we did not see In accumulated at the distal side of wild-type cells located at the proximal border of *in* clones. These data indicate that In accumulates at the proximal side of wing cells. These experiments also provided a control for the specificity of our immunostaining.

fz Pathway Requirements

To determine if the function of the planar polarity (or other) genes was required for the localization of In, we looked in cells mutant for planar polarity genes. We did this both in completely mutant wings and in wings that contained marked clones that were mutant for the relevant gene. This later approach was more compelling as surrounding wild-types cells provided an internal positive control for the staining. We found that mutations in the four planar polarity genes tested in clones (*fz, Van Gogh* [*Vang*] [also known as *strabismus*], *starry night* [stan] [also known as *flamingo*], and *prickle/spiny leg* [*pk/sple*]) blocked the zigzag accumulation of In (Figures 3A–3H). To a variable extent, the In protein was localized to the apical cell periphery in mutant cells, and in some cases, this was uneven, albeit in a random way. The dramatic zigzag pattern was always lost. In some cases, In accumulated at the border of mutant and wild-type cells, but this was not consistent, even within a clone. In addition, in cells bordering *fz* and *Vang* clones, we found that the polarized accumulation of In was often abnormal. The pattern seen was consistent with the hypothesis that this was due to the domineering nonautonomy of such clones [18] as it was seen on the anterior, posterior, and distal boundaries of *fz* clones [19] and the anterior, posterior, and proximal boundaries of *Vang* clones [20]. In some *Vang* and *pk* mutant cells, the level

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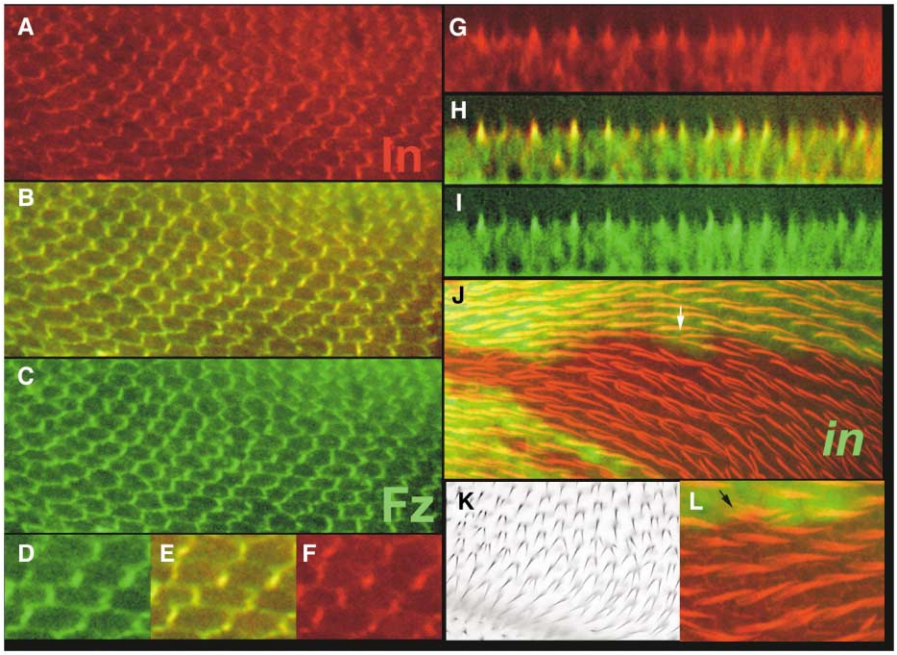

Figure 1. Inturned Accumulates Asymmetrically in Pupal Wing Cells

(A–I) are confocal images of an *arm-fz-GFP* pupal wing stained with anti-GFP (green) and anti-In antibodies (red). (B, E and H) are merges of the single color images. (D, E, and F) are high magnification images of a small section of (A, B, and C). (G, H, and I) are Z section reconstructions. Note the zigzag staining of both Fz and In, the similar unevenness of the staining for the two proteins, and the apically localization in wing cells. (J) shows an *in* clone marked by the loss of GFP in a pupal wing stained with Alexa 568 phalloidin. Note the many multiple hair cells and abnormal polarity of the clone cells. An arrow points to a single wild-type cell that has formed two hairs. (L) is a blow up of this part of (J). (K) shows a small region of an *in* mutant adult wing. Note the abnormal polarity and multiple hair cells. In all images, distal is to the left and proximal to the right.

of In appeared to be increased inside the clone, but this was not always seen. The *grainy head* (*grh*) transcription factor is required for the expression of *stan* in the pupal wing, for the asymmetric accumulation of PP proteins, and for normal wing hair differentiation [21]. We immunostained pupal wings that contained *grh* clones with anti-In antibody and found that *grh* function was also required for the asymmetric accumulation of In. The level of In staining inside of *grh* clones appeared lower (Figures 3K and 3L). This was seen both apically where the PP proteins and In accumulate and basally in the cell (data not shown). The decrease in In was less dramatic but otherwise reminiscent of that seen for Stan.

Mutations in *dachsous* (*ds*) lead to abnormal polarity by causing the *fz* pathway to signal in an anatomically abnormal direction, and it has been suggested that *ds* functions in helping to set up the global direction of *fz* signaling [22–25]. We found that In protein accumulated asymmetrically in *ds* clones. Only in very large clones did we seen any indication that the In pattern was abnormal. These data fit with the previous conclusion that the *fz* pathway is functional in a *ds* clone (Figures 3I and 3J) [22–24].

The directed expression of PP proteins can result in reproducible redirection of hair polarity [26]. For example, when *ptc*-Gal4 is used to drive the expression of *fz* or *stan*, the resulting gradient of expression inside of the patched domain repolarizes hairs so they point either down (*fz*) [26] or up (*stan*) [9] the gradient. Such a treatment also results in the relocalization of PP proteins [4–9, 16]. To determine if the proximal localization of In was instructed by the PP genes, we examined the localization of In in wings where *fz*, *stan*, *Vang*, or *pk* expression was driven by *ptc*-Gal4. We found that In now accumulated along the anterior/posterior boundaries of cells in the *patched* domain much as has been seen for the PP proteins (Figures 3O–3R). This argues that the planar polarity proteins instruct the localization of In.

Loss of function mutations in *fy* and *frtz* results in mutant wing hair phenotypes that are almost identical to *in* and do not disrupt the asymmetric accumulation of PP proteins, and double mutants of these genes have a phenotype that is similar to the single mutants [13, 14]. These observations suggest that these genes function together in planar polarity. We immunostained pupal wings containing marked *fy* or *ftrz* clones and found that the asymmetric accumulation of In protein was lost (Figures 2C–2H). It often appeared that there was not only a loss of asymmetric accumulation but also a reduced level of In protein in mutant cells. We examined the level of In protein by Western blot analysis of wing disc proteins and found a markedly reduced level in *fy* mutants consistent with the suggestion from the immunostaining experiments (Figure 2). We also examined cells that carried mutations in *mwh*. Mutations in *mwh*

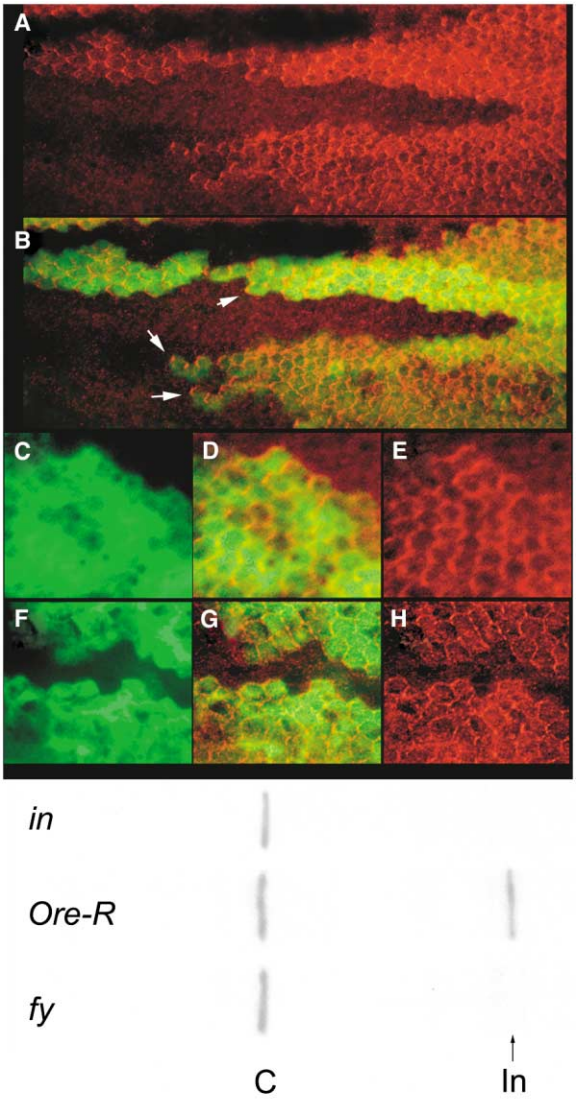

Figure 2. Inturned Accumulates at the Proximal Side of Wing Cells

(A and B) show a large *in* clone marked by the loss of GFP (B, merge) in a wing stained with anti-In antibody (red)(A). Arrows point to wild-type cells at the distal border of the clone that show proximal accumulation of In.

(C, D, and E) show part of a *frtz* mutant clone marked by the loss of GFP (C) stained with anti-In antibody (E) (D, merge).

(F, G, and H) show part of a *fy* mutant clone marked by the loss of GFP (F) stained with anti-In antibody (H) (G, merge). Note the loss of the asymmetric accumulation of In in the *frtz* and *fy* mutant cells.

Shown at the bottom of the figure is a Western blot of wing disc proteins from in mutant, wild-type, and fy mutant larvae. The location of the In protein is indicated. Note that it is lost in the in mutant and in the fy mutant. The band labeled C serves as a loading control. An unknown protein recognized by the batch of secondary antibody is used in the experiment.

are epistatic to mutations in *in, fy*, and *frtz*, and *mwh* is thought to function downstream of *in, fy*, and *frtz* [13]. Consistent with those hypotheses, we found no disruption of asymmetric In accumulation in *mwh* cells (Figures 3M and 3N).

How Does Proximal In Restrict Hair Formation to the Distal-Most Region of a Wing Cell?

It is possible that proximal In activates the cytoskeleton at the distal edge of neighboring cells—i.e., it acts nonautonomously. Previous data suggested that *in* functioned largely cell autonomously [15], however examples were seen where the presence of *in* mutant clone cells resulted in neighboring cells forming more than one hair. In those experiments, the *in* clone was marked by a hair morphology marker. It seemed possible that this was not a truly gratuitous marker, so we reexamined this point by examining pupal wing *in* clones marked by the loss of GFP. Clones smaller than 32 cells in size often did not show a phenotype presumably because of perdurance. Clones larger than 32 cells routinely showed a mutant phenotype (altered hair number and/or polarity) (39/44 clones). Weak domineering nonautonomy was common among larger clones (22/44 clones), however on a cellular basis, it was still very weak, because it typically consisted of only one or two neighboring cells showing a phenotype (Figures 1J and 1L). The location of these abnormal wild-type cells was neither restricted to nor preferentially located on the proximal side of the clone as predicted by the nonautonomous model above. We conclude that proximal In acts within the cell to restrict hair formation to the distal side.

Discussion

The experiments we reported here show that the In protein becomes preferentially localized to the proximal edge of pupal wing cells under the instruction of the upstream PP genes. It remains to be established as to how In is recruited to the proximal side of wing cells. One possibility is that In is recruited to the proximal cortical domain by binding to either Pk or Vang as these proteins accumulate there. Our attempts to detect a direct interaction between In and Pk or Vang by the yeast two hybrid system have not been successful, but such an interaction remains possible. Our finding that the function of *fy* and *frtz* is required for the asymmetric accumulation of In supports the hypothesis that these three PPE genes represent a module that functions to transduce the asymmetric accumulation of PP proteins to the cytoskeleton. Because *mwh* is epistatic to *in*, *fy*, and *frtz*, it would not be surprising if the Mwh protein functioned between In and the cytoskeleton.

The activation of the cytoskeleton to form a hair in the vicinity of the distal-most part of a wing cell is regulated by the PP genes, and it was previously suggested that the basis of this was the local activation/accumulation of Fz and Dsh [4, 10, 11]. The proximal accumulation of Pk and Vang has only been suggested to play a role in the establishment of the proximal and distal cortical domains. Although quite reasonable, it was not easy to test this hypothesis because of the corequirement for all of the PP proteins for their asymmetric accumulation of the others. Our results argue that this hypothesis is incorrect and that at least one proximally localized protein (In) has a role in the distal activation of the cytoskeleton that is unrelated to promoting the distal accumulation of Fz and Dsh.

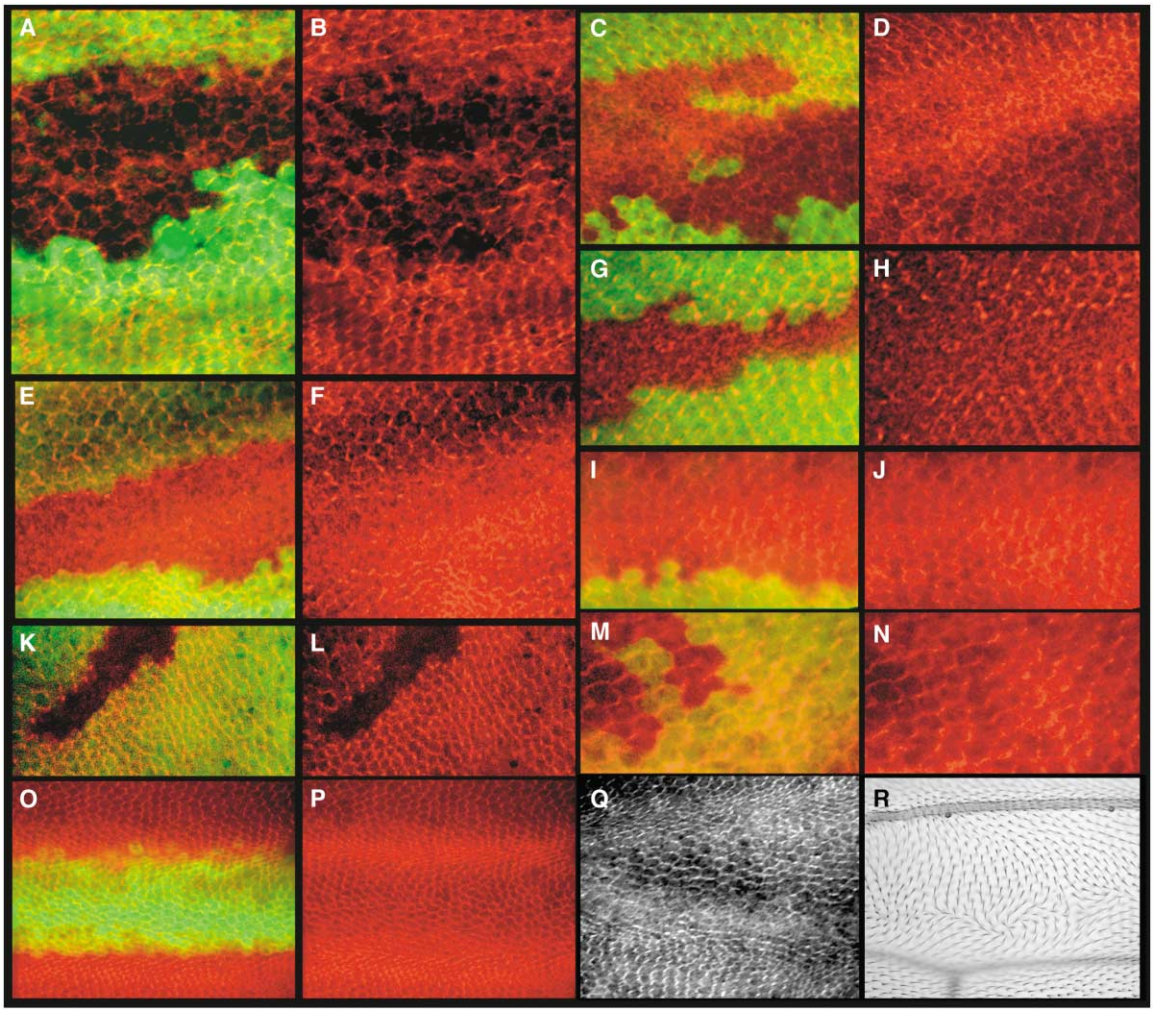

Figure 3. Planar Polarity Genes Instruct Inturned Accumulation

(A–L) show clones of mutant cells marked by a loss of GFP (green) and stained with an anti-In antibody (red). Both merged and anti-In only images are shown. (A and B) show a *fz* clone; (C and D), a *pk* sple clone; (E and F), a *Vang* clone; (G and H), a *stan* clone; (I and J), a *ds* clone; (K and L), a *grh* clone; and (M and N), a *mwh* clone. Note the asymmetric accumulation of In is lost in *fz, pk, Vang, stan*, and *grh* clones. Note that the zigzag staining of In is not altered by the *ds* and *mwh* clones. (O [merged] and P [ant-In antibody]) are of a *ptc-Gal4 UAS-GFP/+;UAS-pk* wing stained with anti-In antibody. (Q) shows a *ptc-Gal4 UAS-GFP/+;UAS-stan* wing stained with anti-In antibody. Note the alteration in the direction of the In staining pattern in response to the directed expression of *pk* and *stan* in (O, P, and Q). (R) shows an adult *ptc-Gal4 UAS-GFP/+;UAS-stan* wing. Note the alteration in the normal proximal to distal polarity of the hairs.

in function is essential for the activity of the PP genes to influence hair formation, and it seems likely that the localized accumulation of In is a key to the mechanism. It is, however, surprising that In is localized on the opposite side of the cell from where the hair forms (Figure 4). How does the recruitment of In to the proximal side of the cell ensure that hairs form at the distal edge? One possibility is that proximal In could stimulate hair formation at the distal edge of neighboring cells. This hypothesis predicts that *in* functions nonautonomously and predicts that we would see a high frequency of abnormal hair formation by wild-type cells located proximal, but not distal, to *in* clones. We failed to see a high frequency of such nonautonomy and hence conclude that a nonautonomy model is unlikely to be a major mechanism by which In functions in wing planar polarity. We did see a low frequency of nonautonomy. The mechanism responsible for this is unclear. One possibility is that although In functions primarily downstream of the PP proteins, there may be some positive feedback where the proximal accumulation of In promotes the asymmetric localization of the PP proteins. This could lead to modest defects in the asymmetric localization of PP proteins that would not be obvious by confocal microscopy but which would be detectable biologically.

We suggest two alternative models by which proximal In could stimulate distal activation of the cytoskeleton within the same cell. It is possible that proximal In functions in the generation of a local inhibitor of hair initiation (or that it sequesters an activator of hair initiation). This could lead to an intracellular gradient of inhibitor biasing hair initiation to the most distal part of the cell. Consis-

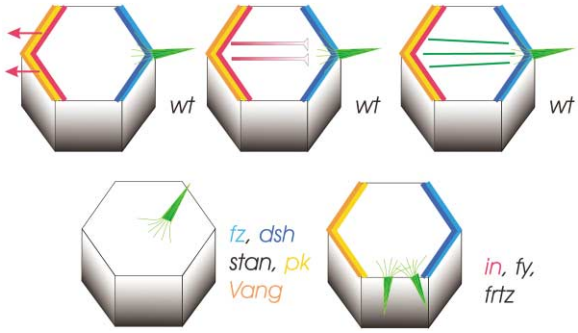

Figure 4. Cartoon of Wing Cells

The hair is shown in green. In the upper row of wild-type cells, the hair forms at the distal-most vertex and points distally. In wild-type cells, Fz (blue) and Dsh (purple) accumulate at the distal side of the cells. Vang (orange), Pk (yellow), and In (red) accumulate at the proximal side of the cells. Three possible mechanisms are shown for how the proximal accumulation of In stimulates the distal formation of the hair. In the cell on the left, proximal In stimulated hair formation at the distal side of proximal neighbor cells. Our mosaic experiments argue that this is not the correct explanation. The cell in the middle shows proximal In resulting in an intracellular gradient of inhibition of hair formation. The cell on the right shows proximal In organizing the intracellular transport of factors involved in hair elaboration. The cells on the bottom show the consequences of mutations in various genes. The cell on the left represents an *fz, dsh, stan, pk*, or *Vang* mutant. None of the PP proteins nor In accumulate asymmetrically, and the hair is formed at a relatively central location on the apical cell surface. The cell on the bottom right represents an *in*, *fy*, or *frtz* mutant where the PP proteins accumulate asymmetrically, but In does not, and multiple hairs form at abnormal locations.

tent with this model is the observation that *in* retains some function in PP mutants where it is not localized properly (i.e., many more multiple hair cells are formed in *in* than *fz* mutant wings [13]). Alternatively, proximal In could organize the polarized intracellular transport of cellular constituents that play a role in hair morphogenesis. The microtuble cytoskeleton is an obvious potential target in this model, however previous observations failed to detect an alteration in the microtubule cytoskeleton in *in* mutants [27].

Experimental Procedures

Fly Genetics

Mutations are described in FlyBase. Fly stocks were either generated in this laboratory, obtained from the *Drosophila* stock center in Bloomington, Indiana, or kindly provided by T. Uemura, T. Wolff, D. Strutt, and J. Axelrod. To generate genetic mosaics, we used the FLP/FRT system. Flp was provided either from an hs-inducible transgene or from *vg-Gal4* and *UAS-flp*. The Gal4 UAS system was used to direct transgene expression. In line with FlyBase usage, we use *Vang Gogh* instead of *strabismus* and *starry night* instead of *flamingo*.

Immunostaining

Immunostaining used monoclonal antibodies directed against In isolated in this laboratory [28]. Staining procedures were standard with the modification of very mild fixation. Previous results with these monoclonal antibodies failed to detect the asymmetric localization of In, because the standard fixation conditions blocked the ability of the antibody to stain endogenous apical In. We routinely removed timed pupae from their pupal case, punctured the pupal cuticle at the head, and then fixed for 1 hr at 4°C in 4% paraformaldehyde, PBS. This limited fixation made the experiments more difficult and led to less than ideal tissue and cell morphology, but it was essential. After fixation, the pupae were rinsed and wings dissected and stained by standard procedures. Secondary antibodies were obtained from Molecular Probes, as was labeled phalloidin.

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